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Isolation, Purification and Screening of Fingal Strain for Effective Bioconversion of Palm Oil Mlll Effluent

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Abstract

A study was conducted to evaluate potential fungal strains isolated from palm oil effluent (POME) sludge Dengkil, Kuala Lumpur Malaysia. A total of 20 fungal strains were isolated for the production of lignocellulolytic enzyme. The isolated strains were purified by conventional techniques and identified by microscopic examination. The strains isolated belong to the genera of *Penicillum*. Six strains were selected for the screening test using POME as media for the production of lignocellulolytic enzyme. During the screening, three parameters were measured to evaluate the potential fungal strain for the production of cellulase and lignin peroxidase (LiP). The parameters were; pH, total suspended solids (TSS) and chemical oxygen demand (COD) were analysed during this study to get the maximum production of lignocellulolytic enzyme. The cellulase production of strain P1-EFB yield 33 filter paper units (FPU/ml) compared to other tested strains, while lignin peroxidase produced 5038 unit enzyme per litre (U/L) of POME sludge. The COD removal was observed in the treatment sludge to be 95% by this strain.

Keywords: Palm oil Mill Effluent, Cellulase, Lignin peroxidase, Penicillium

1.0 Introduction

The widely cultivated oil palm (*Elaeis guineensis*) found in Malaysia is originated from tropical African countries in the late 1870s. It is around 1960s that the rapid production of palm oil began due to the prolonged decline of rubber prices. In fact, it was the government decision to convert large areas of rubber estates into palm oil plantation under the Government Crop's Diversification Programme [1, 2]. Palm Oil Mill effluent is brown thick waste composed of about 2–5% solids, high COD about 47,000 mg/l, less than 1% residual fatty acids, and about 94% water. The organic nitrogen content of the POME sample was less than 1% [3]. The raw POME contains a large fraction of lignin and other molecular weight structural material from the crushed palm oil nut in addition to the lipids and fatty acids from incomplete steam extraction [4]. Palm oil mill effluent (POME) is the mixture of high polluted effluent (from sterilizer and oil room) and low polluted effluent (steam condensate, cooling water, boiler discharge and sanitary effluent) [5]. However, if it is discharge into water courses, the biological reaction will deplete the dissolved oxygen in the water which could affect aquatic life, i.e., fish, prawn and other aquatic animals that provide a significant share of the diet [6].

Bioconversion or bioremediation is a natural process carried out by soil and aquatic microorganism, mostly bacteria and fungi. Certain strains of bacteria and fungi have the ability to breakdown or transform the organic materials present in waste sludge. Bioconversion processes accelerate the biodegradability of wastewater sludge to maximize the break down of organic matter. Condition such as the nature and concentration of co-substrate (s), initial pH, temperature and inoculum size are optimized for biodegradation-bioconversion of waste-water sludge [7]. From the operational and economical point of view, one of the most important steps in biological waste treatment is the separation and the removal of the excess generated during treatment [8]. The most abundant strains isolated from the POME sludge were identified as *Penicillium,* followed by *Aspergillus,* and *Trichoderma*. Although the genus *Penicillium* growth rate is slow, but it produced the highest biomass when grow in supplemented sludge treatments and exhibited the best potential for adaptation.

The better potential of adaptation in sludge environment of *Penicillium* species plays an important role with their ability to convert lignocellulic bulking substances into compost [9]. The two main plant compounds, cellulose and lignin are degraded by both bacteria and fungi. The fungi play the major role because bacteria generally unable to degrade lignin completely as it do not have the lignin-degrading capacity as fungi [10]. Lignin is an important structural component in mature plant material composting 1/3 of wood, and it biodegradability is depend on the presence of oxygen availability. Apparently most degradation of xenobiotic compounds occurs after active growth, during secondary metabolic lignin degradation phase. Degradation of some compounds involves important extracellular enzymes including lignin peroxidase, manganese-dependent peroxidase, and glyoxal oxidase. Cellulose is more difficult to digest; many fungi and a few bacteria produce cellulase that hydrolyzes cellulose to cellobiose and glucose [11]. The potentiality of *Penicillium* species in well-controlled condition and found that *Penicillium* species produced cellulases and hemicellulases which had some effects on the carbon source during enzyme production [12] is one among the promising factors to exploit its potential in this study.

2.0 Materials And Methods

2.1 Sample Collection

The samples were collected from four different sources of oil palm waste which comprises compost, empty fruit bunch, POME and treatment pond of Seri Ulu Langat Oil Palm Sdn. Bhd., Dengkil, Selangor.

2.2 Isolation and Purification

The Rose Bengal Agar (RBA) medium was used for isolation of *Penicillium* spp [13] with some modification [14, 15]. The isolation medium contained (g per liter): KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, peptone 5.0, dextrose 10.0, Rose Bengal agar 0.35, Agar 10.0 and streptomycin 2 ml (50 mg/l). All compositions were dissolved in distilled water except streptomycin and autoclaved at 121^{o}C for 15 min. Streptomycin was added to liquid media before solidification state. One milliliter of sample and 15 ml of liquid media were poured into the sterile Petri dishes and

incubated at room temperature (30°C±2°C) and allowed to grow for 3-4 days. Then single well developed colony was picked on Potato Dextrose Agar (PDA) plates and subcultured to purification. Pure cultures of filamentous fungi were obtained after successive transfer of individual colony in PDA plates and incubated for 3-7 days at room temperature.

2.3 Preparation of the Inoculum

Inoculum preparation (spore suspension) was done according to method [15]. The cultures were grown in PDA medium in petri dishes at 32^{o}C for 7 days. Then the petri dishes were washed with 100 ml of sterile distilled water. The surface was gently rubbed with a sterilized hockey stick and the mycelial suspension was transferred into sterilized 250ml of Erlenmeyer flask by filtration. It was then shaken in a rotary shaker with 150 rev/min for 24 hours. The fungal growth as suspension was collected through filtration by Whatman # 1 filter paper. For inoculum verification process, 2% of malt extracts (ME) as co-substrate was added into 50 ml of distilled water in a 250ml of Erlenmeyer flask. The solution autoclave at 121^{o}C for 15 min before added with 1 ml of the inoculum. The flasks then placed on the rotary shaker at 150 rev/min in room temperature for 1-2 days.

*2.4 Identification *

The cultures were grown on PDA (3.9% w/v) in Petri dishes at three points in the case of slow growing fungi (*Penicillium*) and its growth rate, odour, colour and changes in media colour after five days incubation were observed. The morphology of the isolates was determined by using an Image Analysis System, consisting of a microscope, a CCD camera, a personal computer and image analysis software (Olympus Micro Image Lite 4.0). It results were compared by the references to classical keys [16]. Identification was done by examining the size and shape of phialospores and condiospores, the shape and arrangement of phialides, colour, appearance and odour of mycelia on the plates.

2.5 Lignin Peroxidase Assay

The standard activity of LiP was measured on the basis of oxidation reaction of Veratryl alcohol in the presence of hydrogen peroxide [17]. A 600 µL Veratryl solution (10mM) was mixed with 1.50ml of distilled water, 50 of LiP sample solution, and 600 L of pH 2.5 tartrate buffer (0.25M). The absorbance of the veratraldehyde was measured at 310 nm after 1 minute added with 240 µL of 5mM H_2O_2.The absorbance of the product, veratraldehyde was 9300 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount enzyme generating of 1 µmol of veratraldehyde per minute under the described reaction conditions.

2.6 Cellulase Assay

The cellulase activity was measured according to the method [18]. The procedure has been designed to measure cellulase activity in term of “filter-paper units” (FPU) per millilitre of original (undiluted) enzyme solution. Thus the conversion of the enzyme cellulase activity based on the linear glucose standard curve that constructed using the absolute amounts of glucose

(mg/0.5ml) plotted against A_{540}. The correlation of the standard glucose curve must be very near to one.

2.7 Analytical Methods

The fungal growth was measured as the total suspended solids in the treatment. The TSS and COD were determined according to the standard methods (APHA 1989) [19]. The pH was recorded in supernatant of treated sludge. The data were the average of three replicates.

3.0 Results and Discussion

3.1 Isolation and Purification

A total of twenty strains of *Penicillium* species were isolated from four different sources of POME sludge which comprises compost, empty fruit bunch, POME and treatment pond of Seri Ulu Langat Oil Palm Sdn. Bhd., Dengkil, Selangor. However, only 6 strains were selected for further screening to identify the best strain of filamentous fungi for the production of lignin peroxidase and cellulase using POME as the substrate of the *Penicillium* (P_1-EFB, P_2-EFB, P_3-EFB, P_4-EFB, P_5-EFB and P_6-Pond) as shown in Figure 1. Those remaining were discarded based on their comparatively poor growth in PDA culture. All isolated strains were identified based on their mycelial growth, colour, changes of medium colour and the morphological characters (Figure 2). The colour observed among the strains P_1-EFB, P_2-EFB, P_4-EFB and P_6-Pond were ash green, light green, and dark green at the centre and surrounded with a compact vertical phailides. While the colour of strains P_3-EFB and P_5-EFB, were light white. The P_3-EFB showed a ring with light green colour and the inner part and outside of the ring was whitish in colour. The P_5-EFB appeared to be a scar-like shape. Both P_3-EFB and P_5-EFB have shown slower growth rate compared to the other strains. The reverse plates of the cultures were slightly changed in colour for few isolates which were yellowish, creamy and pale green for P_1-EFb, P_2-EFB, P_4-EFB, P_5-EFB and P_6-POND. For P_3-EFB, the reverse plate was distinct yellowish in colour with scar-like shape.

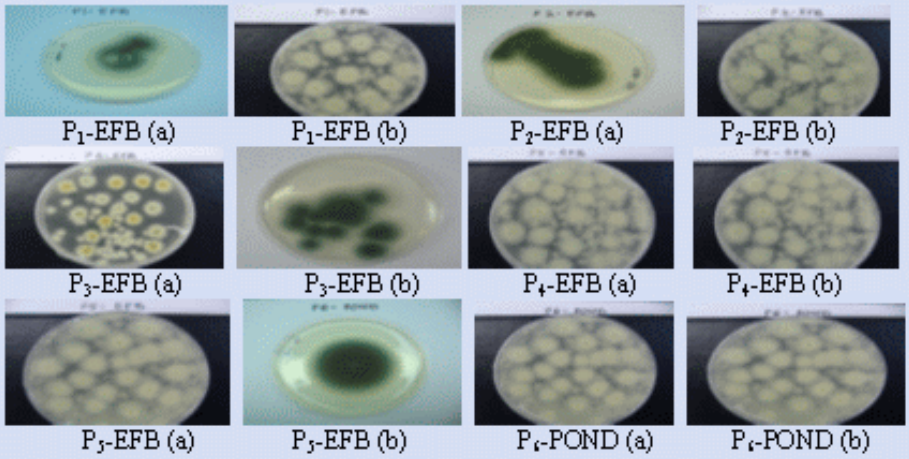

Figure 1 : 6 strains of Genus *Penicillium* growing on PDA at room temperature; (a) the front view of single colony of the strains after 7 days cultures; (b) the reverse view of the strains after 3 days cultures. EFB-isolated from empty fruit bunch; POND-isolated from oxidation pond sludge.

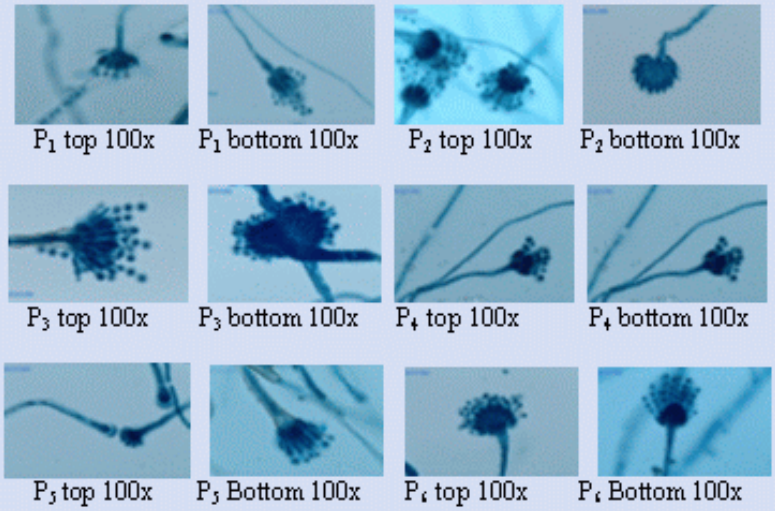

Figure 2 : Reproductive structure of *Penicillium* species isolated from the POME

3.2 Screening of Penicillium

Screening of Penicillium against POME as substrate for lignocellulolytic enzyme production was conducted by observing various physico-chemical parameters for the evaluation of strain performance in liquid state bioconversion (LSB) process. The total suspended solid, pH, lignin peroxidase and cellulase production were observed. Figures 3-6 illustrated the results of the 6 strains of *Penicillium* after 6 days of fermentation treated with 4 parameters for screening process.

Figure 3 showed the lignin production of the strains as the fermentation days increased. It was also noticed that the production of lignin peroxidase by the strain P_1-EFB was greater compared to the other strains. The amount of enzyme was produced 4224 unit per litre on day 2, then slightly decline to 4129 unit per litre in day 4 but drastically increased to 5038 unit per litre in day 6. While the production of lignin peroxidase was low and increased gradually by other strains. P_2-EFB produced lignin enzyme 2773 U/L in day 2, 1959 U/L in day 4 and increased to 2080 U/L in day 6. On the other hand, P_3-EFB generated 1859 U/L in day 4 and reduced to 1737 U/L in day 4, but increased back in day 6 to 1786 U/L. For strain P_4-EFB the lignin enzyme produce was 1412 U/L in day 2 and increased to 2355 U/L in day 4 and 2386 U/L in day 6. Whereas, the strain P_5-EFB showed the lignin peroxidase 1793 U/L in day 2 which reduced to 1424 U/L in day 4 and increased to 1735 in day 6. The lignin enzyme produced was 1535 U/L in day 2, increased drastically in day 4 to 1950U/L and 2123 U/L in day 6 by the strain P_6-Pond.

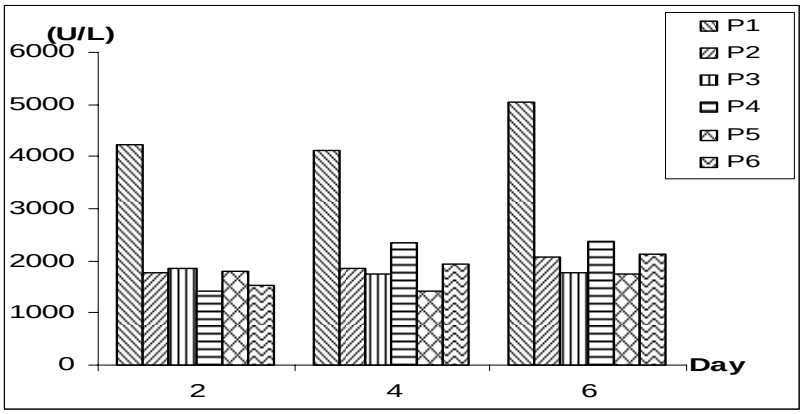

Figure 3 Ligninase peroxidase production of the 6 *Penicillium* strain isolated from POME

The values of the cellulase production by fungal treatment were illustrated in Figure 4. The production of the cellulase by P_1-EFB strain showed the best production especially on day 6 among the strains. The production was 33 filter paper units significantly high compared to other tested strains. The cellulase productions for P_1-EFB were 22 and 21 FPU at day 2 and 4 respectively. While the cellulase productions for strain P_2-EFB showed 21 FPU in day 2; 13 and 6 FPU in day 4 and 6 respectively. For strain P_3-EFB, the FPU production increased to 10 FPU in day 4 compared to 3 FPU in day 2, it then reduced to 8 FPU in day 6. For P_4-EFB the productions of cellulase significantly reduced from 13 FPU to 7 FPU from day 2- 4. While it was 3 FPU in day 6 for strain P_4-EFB. The cellulase production of P_5-EFB increased to 18 FPU in day 4 compared to 12 FPU in day 2, but decreased drastically to 4 FPU in day 6. For strain P6-Pond, the cellulase production was 24 FPU in day 2 while reduced to 17 FPU in day 4 and 15 FPU in day 6 respectively.

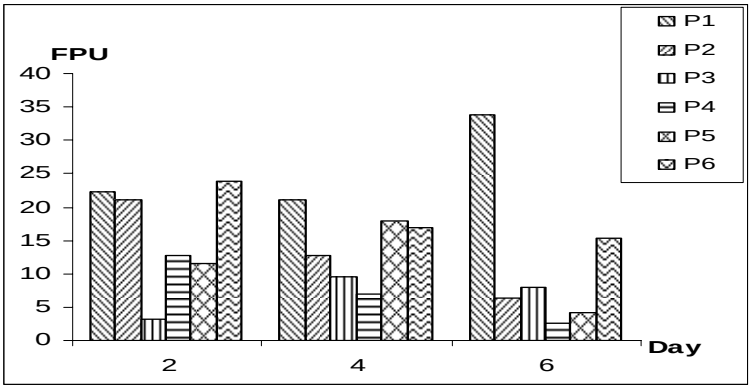

Figure 4 Cellulase production of the 6 *Penicillium* strains isolated from POME

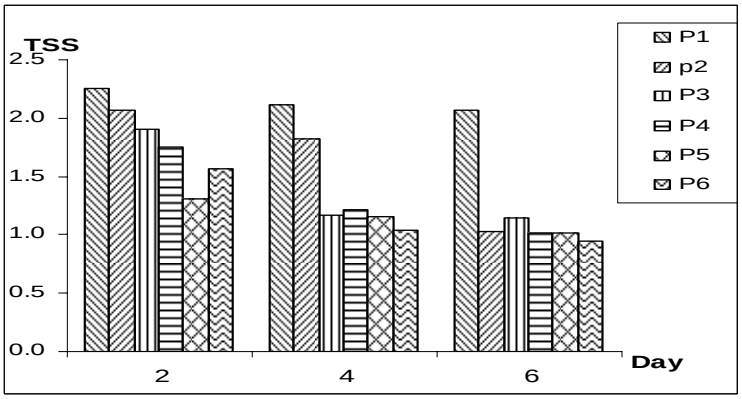

Figure 5 Total Suspended Solid (TSS) values of the 6 *Penicillium* strains isolated from POME

In Figure 5 the effect of the microbial treatment on the total suspended solid (TSS mg/L) of POME for P_1-EFB shown that the reduction was gradually decreased to 2.25%, 2.12% and 2.07% in day 2, 4 and 6 respectively. While, the strain P_2-EFB was drastically decreased from 1.82% in day 2 to 0.91% in day 6. The strain P_3-EFB showed gradual reduction of TSS from day 2 to day 4 (1.90% to 1.17%) but slightly reduced 1.14% in day 6. For strains P_4-EFB and P_5-EFB, the TSS reductions were slightly different compared to day 2 which were 1.75% and 1.31% respectively. The strain P_6-Pond showed TSS reduction from day 2 with 1.57% reduction compared to 1.04% reduction in day 4 and 0.95% in day 6. Figure 5 also showed that all strains were decreasing in tendency as the fermentation time increased.

The values of pH are illustrated in Figure 6. Almost all the strains showed the tendency of increasing pH value as the fermentation time was increased. The pH values varied from 4.03 to 7.83 at 2, 4 and 6 days of treatments respectively. In fact, the optimum pH for the fungal growth varied with strain or species and the nutritional environment. Initially the pH value was measured for all the strains and was set at pH 4.5 before the fermentation time. This initial pH

was also reported as the best initial pH for bioconversion of domestic wastewater by different fungi [20].

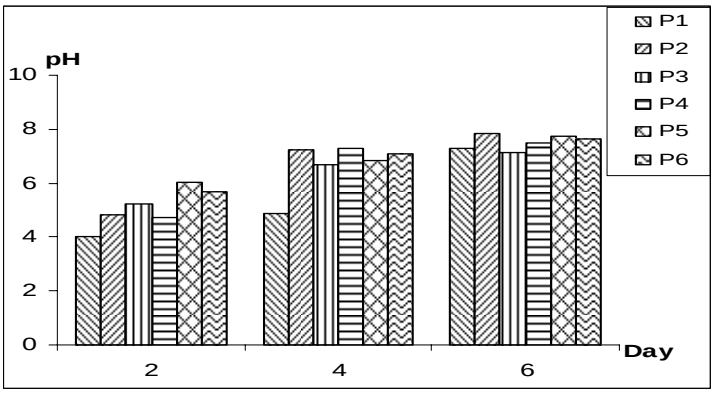

Figure 6 pH values of the 6 *Penicillium* strain isolated from POME

Besides, pH 4.5-5.5 also influences the colour removal [21]. The colour of the samples became darker compared to the control (uninoculated) strain after the fermentation day. This might be due to the increase in pH value as the fermentation day was increased. The highest pH value was observed on day 6 which belongs to strain P_2-EFB while the lowest pH value was on day 2 for strain P_1-EFB.

4.0 Conclusion

The result revealed that P_1-EFB could be the best potential strain for biodegradation in liquid state bioconversion of palm oil mill effluent. The best productions of cellulase and lignin peroxidase enzymes were observed on day 6 by P_1-EFB which showed significantly high production. The cellulase production of strain P_1-EFB yield 33 filter paper units (FPU/ml) compared to other treated strains, while lignin peroxidase produced 5038 unit enzyme per litre (U/L) of (1% w/w) POME sludge. All the strains showed the potential production of both enzymes in acidic condition. The optimum pH for production of those enzymes by strain P_1-EFB was at pH 7.3. Besides, TSS reduction by P_1-EFB was also the highest among the other strains and the maximum removal of COD was 95% by P_1-EFB *Penicillium* strain after 7days of treatment. Thus the findings of this research could provide an alternative environmental biotechnological approach for future research in waste management through value added products and it might contribute to the nation's revenue.

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